Targeted Differential Display

This method was successful in our lab using prostate tissue and for our specific objectives. Investigators must be aware that they will need to tailor the following protocol for their own research objectives and tissue under study.

The protocol below is **one example** of differential gene expression analysis of cells obtained from microdissected tissue.

1. Materials

1. Stratagene RAP-PCR kit
2. 5X RT buffer (GenHunter)
3. dNTP, 250 µM (GenHunter)
4. dNTP, 25 µM (GenHunter)
5. RNase inhibitor (Perkin Elmer)
6. Random hexamer primers (Perkin Elmer)
7. MMLV reverse transcriptase, 100 units/µl (GenHunter)
8. 10X PCR buffer (Perkin Elmer)
9. AmpliTaq DNA polymerase (Perkin Elmer)
10. DEPC- treated H_2O
11. 3 M Na acetate, pH 5.2 (Life Technologies)
12. 100% ethanol
13. 2X loading dye (95% formamide, 20 mM EDTA, 0.05% bromophenol blue, 0.05% xylene cyanol)

2. Methods (Under sterile, RNase free conditions)

Note: The protocol was utilized to screen for tumor-specific alterations in transcript levels using a variety of screening approaches and primer combinations, including the RAP-PCR arbitrary primers from Stratagene and degenerate primers directed against known protein motif DNA sequences. Exact conditions must be determined for each differential display study depending on the number of cells utilized, the primer sets, and the number of transcripts the investigator wishes to screen.

A: Microdissection and RNA Isolation

1. Dissect approximately 5000-10,000 cells from frozen sections cut to a thickness of 12 µm. Complete the microdissection within 30 minutes of preparation of the tissue sections.

2. Isolate total RNA, as from protocol in RNA-based Studies of Microdissected Tissues.

   TIP: Only use cells from frozen tissue that is extremely well-preserved.

   TIP: If anchored, oligo-dT-based differential display is to be performed (e.g., with the GenHunter kit), it is recommended to start with RNA recovered from a substantially larger number of microdissected cells.

B: Reverse Transcription

1. Add 24 µl H_2O and 1 µl RNase inhibitor, 20 U/µl, to RNA pellet.

2. Resuspend the RNA pellet with gentle tapping.

3. Quick spin.

4. Aliquot 12 µl into 2 tubes for the (+) and (-) RT reactions.

5. Prepare additional control tubes using the following in place of the sample RNA:

   - A positive control prepared with known, high-quality RNA
   - A negative control prepared from either a LCM cap containing no tissue or RNase-free water

6. Prepare sufficient volume of the following reaction mixture for each tube:

Reaction mixture/tube	
4 µl	5X RT buffer
2 µl	dNTP mix (250 µM)
1 µl	Random hexamer primers
	Total volume = 7 µl

7. Add reaction mixture to each tube in #3 and #4.

8. Incubate 10 minutes at 25°C.

9. Add 1 µl MMLV reverse transcriptase to the (+) RT tube and the positive and negative controls. Add 1 µl water to the (-) RT tube.

10. Incubate 10 minutes at 25°C.
11. Heat 40 minutes at 37°C.
12. Heat 5 minutes at 95°C.
13. Store the cDNA at -20°C until use.

C: PCR

> **TIP:** Investigators must be especially careful when using this methodology to analyze archival tissue specimens. Formalin fixation in particular results in DNA that is difficult to amplify and often produces inconsistent PCR results, including artifactual allelic loss and poor amplification of large products. If this technique is to be utilized for analysis of archival samples, we highly recommend that replicate experiments (multiple independent dissections, triplicate PCR reactions, etc.) be used to verify results.

1. Prepare sufficient volume of reaction mixture for all tubes:

Reaction mixture/ tube	
1.0 µl	10X PCR Buffer
0.8 µl	dNTP mix (25 µM)
0.2 µl	Forward primer, 25 µM (see **TIP 1**)
0.2 µl	Reverse primer 25 µM
0.2 µl	^{33}P or ^{32}P, 20 mCi/ml,
0.2 µl	AmpliTaq DNA polymerase, 5 U/µl
6.9 µl	dH_20
	Total volume = 8.5 µl

2. Add 1.5 µl cDNA (or DNA) from #13 to each tube.
3. Add reaction mixture to each tube.
4. PCR cycling conditions in a PE 9600 thermocycler (see **TIP 2**)

Cycles	Temp. (°C)	Time (min)
1	94	2
35	94 50 72	0.5 0.5 2
1	94	2

5. **TIP 1:** In the published work cited above, degenerate primers directed against zinc finger motifs were used in conjunction with Stratagene's RAP-PCR arbitrary primers. To generalize this protocol, the PCR primers are written as forward primer and reverse primer. Please refer to the Stratagene protocol for more details.

   TIP 2: The times and temperatures given above are samples only and what was used in the above referenced work. Please refer to the RAP-PCR protocol for details regarding the PCR conditions. We tried annealing temperatures varying from 50-58°C.

D: P.A.G.E.

While the PCR is cycling, pour a 6% polyacrylamide sequencing gel:

1. After cycling is completed, add 10 µl 2X loading dye to samples.
2. Denature samples at 95°C for 3 minutes then place directly on ice.
3. Load 3.5 µl sample on gel.
4. Run at 55W until the xylene cyanol migrates to the bottom of the gel.
5. Transfer gel to 3 mm Whatman paper and dry.
6. Expose to film in cassette with intensifying screen.
7. Develop film.
8. Look for differentially expressed bands between samples.

E: Sequencing of Differentially Expressed Bands

9. Excise the appropriate bands from the gel using a razor blade.
10. Place the piece of gel/paper in a microfuge tube.
11. Add 100 µl water.
12. After 2-5 min. peel the acrylamide off the paper.
13. Place the acrylamide in a new tube.
14. Grind the acrylamide with a pipet tip.
15. Add 100 µl water.
16. Heat at 94°C for 10 min.
17. Precipitate the DNA using 0.1 x volume 3 M sodium acetate, and 2.5 x volume 100% ethanol.
18. Resuspend the DNA pellet in 40 µl water.
19. To 12.2 µl DNA from #10 above, add the following re-amplification reagents:

Reaction mixture/ tube	
2.0 µl	10X PCR buffer
1.6 µl	dNTP 25 µM
2.0 µl	Forward primer, 25 µM
2.0 µl	Reverse primer, 25 µM
0.2 µl	AmpliTaq DNA polymerase
	Total volume = 7.8 µl

20. Use the same cycling conditions as in the first PCR, #2 above.

21. Run out the PCR product on a 1.5% low melting point agarose gel.

22. Cut out the PCR product (approx. 100 µl) and use and aliquot directly in the sequencing reaction.

23. Sequence according to manufacturer's instructions. In the paper referenced below, we used the Perkin Elmer Amplicycle Sequencing kit.

F: Reference

The protocol was taken from:
Chuaqui et al, Identification of a novel transcript up-regulated in a clinically aggressive prostate carcinoma. J Urol 50(2):302-7, 1997.